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PRINCIPAL INVESTIGATOR: Jeffrey D. Singer, Ph.D.
James M. Roberts, Ph.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, Washington 98104-1024

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6. AUTHOR(S)
Jeffrey D. Singer, Ph.D.
James M. Roberts, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center
Seattle, Washington 98104-1024

E-MAIL:
Jsinger@fhcrc.org

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13. ABSTRACT *(Maximum 200 Words)*

Cyclins are proteins that both activate cyclin-dependent kinases, enzymes that put phosphate groups on proteins, and impart some degree of substrate specificity to these kinases. Although very little is known about what these substrates are, it is clear that the regulation of levels of cyclins is crucial for cell cycle progression, that is cell growth. The human cyclin E protein has been shown to be required for the G1/S transition, a crucial regulatory step in mammalian cells that determines if a tumor can form. Cyclin E protein levels have been shown to cycle as the cell cycle progresses. The goal of this work was to identify the components that are responsible for the regulation of the cyclin E protein levels. Such regulation has many important implications for the study of cell cycle progression and thus the onset of tumorigenesis which is an example of uncontrolled cell cycle progression. The result of this work was the identification of a protein in mammalian cells that is responsible for removing cyclin E from cells by directing its degradation. Thus understanding the regulation of this novel protein may lead to insights into how tumors can form in mammals.

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INTRODUCTION

Cyclins have been shown to be proteins that both activate cyclin-dependent kinases and impart some degree of substrate specificity to the cyclin-CDK holoenzyme. Although very little is known about what these substrates are, it is clear that the regulation of levels of cyclins is crucial for cell cycle progression. In the yeast *Saccharomyces cerevisiae* several cyclins have been identified. Some, the CLNs, appear to be specific for the G1/S transition while others, the CLBs appear to be specific for the G2/M boundary. The human cyclin E gene was cloned based on its ability to complement a yeast strain with mutations in its G1 cyclin genes CLN1-3. The cyclin E protein has subsequently been shown to be required for the G1/S transition in mammalian cells. Protein levels have been shown to cycle as the cell cycle progresses such that levels of cyclin E are maximal in early S phase and then rapidly decline. The goal of the work proposed here is to identify the components that are responsible for the regulation of the cyclin E protein levels. Such regulation has many important implications for the study of cell cycle progression and thus the onset of tumorigenesis which is an example of uncontrolled cell cycle progression. Recently, elevated levels of cyclin E protein have been correlated with severity of breast cancer.

BODY

The following three aims were approved in the Statement of Work:

Specific Aim 1) Are proteins that physically interact with cyclin E protein involved in its degradation?

Specific Aim 2) Can an *in vitro* system for regulation of cyclin E be developed by identifying the proteins that regulate ubiquitination of cyclin E?

Specific Aim 3) Are the human homologues of the proteins that regulate yeast G1 cyclins responsible for the degradation of human cyclin E?

The first aim has been addressed during the first year of support. A yeast 2 hybrid screen was utilized to identify proteins that bind cyclin E. The results of the two hybrid screen and subsequent analysis of the protein that was identified has been accepted for publication by the journal Genes and Development and appeared in the September 15 issue.

KEY RESEARCH ACCOMPLISHMENTS (First year)

- Identification of a cyclin E binding protein that is homologous to known cyclin degradation proteins
- Cloning of the cul-3 gene and development of reagents to study its role in the degradation of cyclin E in cells. These reagents include antibodies, cells lines, expression vectors and knock out mice.
- Extensive analysis of knock out mice (still under way).

The second aim was addressed during the second year of support. Baculo expressed cyclin E bound to its kinase CDK2 were made and the requirements for phosphorylation of cyclin E and disassociation of the complex were studied. In addition further analysis of the cul-3 protein were performed by the construction of a conditional knockout of the cul-3 gene. During the analysis of cul-3 other binding proteins were identified which led to an analysis of cul-3 binding to the CDK2 inhibitor p21. These data were published this year in Molecular Cell in February (see appended copy of paper).

KEY RESEARCH ACCOMPLISHMENTS (Second year)

- Construction of a conditional knockout of the cul-3 gene in mice
- 2-hybrid screens using the cul-3 gene as bait to identify components of the degradation complex
- development of a *in vitro* system to study the cyclin E-CDK2 interaction

REPORTABLE OUTCOMES

Part of the data obtained during the second year of support appeared in the February issue of Molecular Cell.

I am applying for a faculty position with the data reported here as the basis for my future work.

CONCLUSIONS

Based on what we have learned so far about the role of cul-3 in the degradation of cyclin E in mammalian cells we are persuing several studies to determine if cul-3 can prevent unwanted cell growth in normal organs. In addition we will use the antibodies we have developed that recognize cul-3 to analyze breast tumors samples for misregulation of cul-3 protein.

APPENDICES

The following is a copy of the paper that appeared in Molecular Cell.

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Proteasomal Turnover of p21^{Cip1} Does Not Require p21^{Cip1} Ubiquitination

Robert J. Sheaff,[1,2] Jeffrey D. Singer,[2] Jherek Swanger,[3] Matthew Smitherman,[3] James M. Roberts,[2,5] and Bruce E. Clurman[3,4,6,7]
[1]University of Minnesota Cancer Center and Department of Biochemistry
University of Minnesota
Minneapolis, Minnesota 55455
[2]Division of Basic Sciences
[3]Division of Clinical Research
[4]Division of Human Biology
[5]Howard Hughes Medical Institute
Fred Hutchinson Cancer Research Center
1100 Fairview Avenue North
[6]Department of Medicine
University of Washington
Seattle, Washington 98104

Summary

The Cdk inhibitor p21^{Cip1} is an unstable protein. Pharmacologic inhibition of the proteasome increases the half-life of p21 from less than 30 min to more than 2 hr and results in the accumulation of p21–ubiquitin conjugates. To determine whether ubiquitination was required for proteasomal degradation of p21, we constructed mutant versions of p21 that were not ubiquitinated in vivo. Remarkably, these mutants remained unstable and increased in abundance upon proteasome inhibition, indicating that direct ubiquitination of p21 is not necessary for its turnover by the proteasome. The frequently observed correlation between protein ubiquitination and proteasomal degradation is insufficient to conclude that ubiquitination is a prerequisite for degradation.

Introduction

The periodic expression of cell cycle proteins is a central feature of cell cycle control. Targeted proteolysis by the ubiquitin–proteasome system has emerged as a key determinant of this periodicity (King et al., 1996a; Peters, 1998; Koepp et al., 1999). Ubiquitin conjugation regulates diverse biological systems and occurs in a series of highly regulated enzymatic reactions, thereby allowing precise substrate targeting in appropriate physiologic contexts (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Several criteria are used to determine if a specific protein is degraded by the ubiquitin–proteasome system: the decrease in the rate of substrate turnover in cells caused by inhibition of the system with chemical inhibitors or by genetic mutations; detection of ubiquitin-substrate conjugates in cells, usually following proteasome inhibition; reconstitution of ubiquitination and/or proteasomal degradation of substrates in cell extracts in vitro; and association of putative substrates with ubiquitinating enzymes.

[7]To whom correspondence should be addressed at the Fred Hutchinson Cancer Research Center (e-mail: bclurman@fhcrc.org).

Many mammalian (e.g., cyclins [E,D,A,B] p27, p21, E2F, Rb, and p53) and yeast (e.g., Clns, Clbs, Sic1, and Far1) cell cycle proteins are ubiquitinated, and their degradation is dependent upon the proteasome in vivo (King et al., 1996a; Peters, 1998; Koepp et al., 1999; and references therein). In some cases (B-type cyclins, p27, Sic1, Far1, and Cln2), ubiquitination and/or proteasomal degradation have also been demonstrated in extracts that recapitulate features of cellular regulation. Furthermore, in yeast cells the degradation of proteins such as Far1 and Sic1 requires specific ubiquitination enzymes, suggesting that turnover of these proteins is directly regulated by ubiquitination. Nevertheless, it is difficult to demonstrate that direct ubiquitination of a given protein is a prerequisite for its degradation in vivo, and in most cases, particularly for mammalian proteins, this has not been shown.

Results

p21 Is an Unstable Protein that Exhibits Proteasomal Turnover and Is Ubiquitinated In Vivo

p21 belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors and mediates cell cycle arrest in response to stimuli such as activation of the p53 tumor suppressor (Sherr and Roberts, 1995). p21 meets at least two of the criteria for ubiquitin–proteasome regulation in that proteasome inhibition results in both p21 accumulation and appearance of p21–ubiqutin conjugates in vivo (Blagosklonny et al., 1996; Maki and Howley, 1997; Cayrol and Ducommun, 1998; Rousseau et al., 1999). Treatment of human diploid fibroblasts with two chemically distinct proteasome inhibitors (the peptide aldehyde MG-132 or the more specific proteasome inhibitor clasto-lactacystin-β-lactone) resulted in the accumulation of endogenous p21 protein (Figure 1A). This was due solely to increased p21 stability. Its half-life increased from less than 30 min to greater than 2 hr (Figure 1B) whereas its rate of synthesis remained unchanged (Figure 1A). In contrast, a radiomimetic dose of actinomycin D, which induces p53, increased the rate of p21 synthesis, but not its half-life (Figure 1A).

Although very low abundance p21–ubiquitin intermediates can be detected in some cell types, we did not detect ubiquitinated endogenous p21 in human diploid fibroblasts after proteasome inhibition, even though p21 was dramatically stabilized. It can be difficult to detect ubiquitin–protein conjugates in vivo due to rapid removal of the ubiquitin chains by deubiquitinating enzymes. We therefore utilized exogenously expressed p21 to assess the role of ubiquitination and specific amino acid residues in p21 turnover. The half-life of transfected p21 was also less than 30 min, and its abundance increased by proteasome inhibition (Figures 1C and 1D). In this case, however, p21–ubiquitin conjugates were readily detected (Figure 1D). These high–molecular weight species contained both p21 and ubiquitin as shown by their altered molecular weight when an HA epitope–tagged ubiquitin molecule was coexpressed with p21 (Figure 1D).

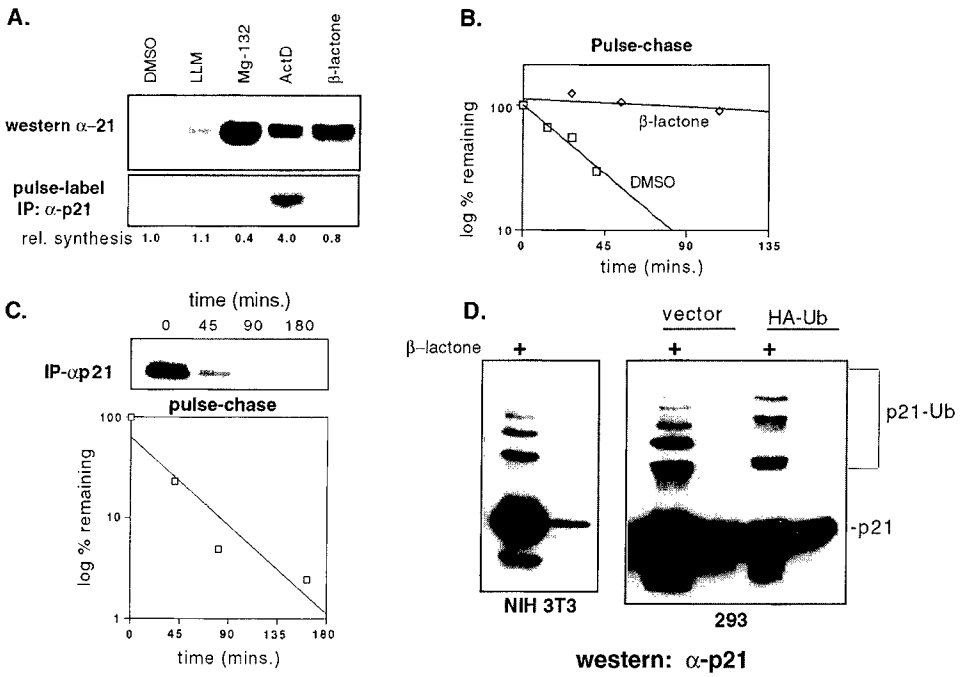

Figure 1. Proteasomal Turnover and Ubiquitination of p21

(A) Human diploid fibroblasts were treated with the indicated drugs for 16 hr (DMSO, LLM, Mg-132, and actinomycin D) or 5 hr (β-lactone) and then pulse labeled for 10 min. Top panel, Western analysis of p21 abundance; bottom panel, amount of p21 protein synthesized during pulse.

(B) Pulse–chase analysis of endogenous p21 in human diploid fibroblasts ± β-lactone.

(C) Pulse–chase analysis of transfected p21 protein in 293 cells.

(D) Transfected p21 is proteasome dependent and ubiquitinated. Left, NIH 3T3 cells were transfected with CS2p21 and treated with β-lactone or solvent control. Right, 293 cells were cotransfected with p21 expression vector and either HA-ubiquitin or control vector, and treated with β-lactone as shown. Ubiquitinated p21 species are shown (p21-Ub).

p21 Protein that Cannot Be Ubiquitinated Remains Unstable

If p21 ubiquitination is a prerequisite for its proteasomal turnover, then inhibition of the ubiquitination pathway should stabilize p21. We tested this hypothesis using four different approaches, all of which suggested that proteasomal degradation of p21 did not require p21 ubiquitination. First, we utilized a ubiquitin mutant (UbR7) lacking lysines that inhibits multiubiquitination of cyclin B, but not monoubiquitination (T. McGarry, personal communication). As a control, cotransfection of this mutant ubiquitin increased the steady-state abundance and half-life of cyclin E, a substrate of the cullin family of E3 ubiquitin ligases (Clurman et al., 1996; Won and Reed, 1996; Dealy et al., 1999; Singer et al., 1999; Wang et al., 1999) (Figure 2A). In contrast, overexpression of UbR7 did not increase the abundance of p21. These data suggest that direct multiubiquitination is required for cyclin E degradation, but not for p21, although we cannot rule out the possibility that cyclin E and p21 are differentially sensitive to conjugation with UbR7.

Second, we mutated all six lysines in p21 (the sites of potential ubiquitin conjugation) to arginine (p21K6R). p21K6R bound, inhibited, and was phosphorylated by cyclin–Cdk complexes like wild-type p21, indicating that its structure and activity were not severely affected by these six conservative substitutions (Figure 2B). No p21–ubiquitin conjugates of p21K6R were detected in vivo after proteasome inhibition in either NIH3T3 or 293 cells, even when highly overexpressed and when the film was overexposed (Figure 2C and data not shown), indicating that ubiquitination of p21 was prevented by mutation of the lysines. Surprisingly, p21K6R not only remained unstable, but its abundance also increased in response to proteasome inhibition indistinguishably from wild-type p21 (Figure 2C). Furthermore, the half-life of both wild-type p21 and p21K6R increased from less than 30 min to greater than 2 hr after proteasome inhibition (Figure 2D).

In parallel, we also examined the degradation of a lysineless mutant of cyclin E (cyclin EΔK). Mutating all of the lysines in cyclin E prevented formation of cyclin E–ubiquitin conjugates (Figure 2E). However, unlike p21, the abundance of cyclin EΔK was no longer responsive to proteasome inhibition, consistent with a major role for ubiquitination in cyclin E turnover.

Third, we examined the role of p21 ubiquitination under more physiologic conditions utilizing stable retrovirally transduced cell lines. The retroviral p21 was of human origin and introduced into mouse cells, allowing us to specifically monitor expression of the exogenous and endogenous p21 proteins by using species-specific antibodies and the different electrophoretic mobilities

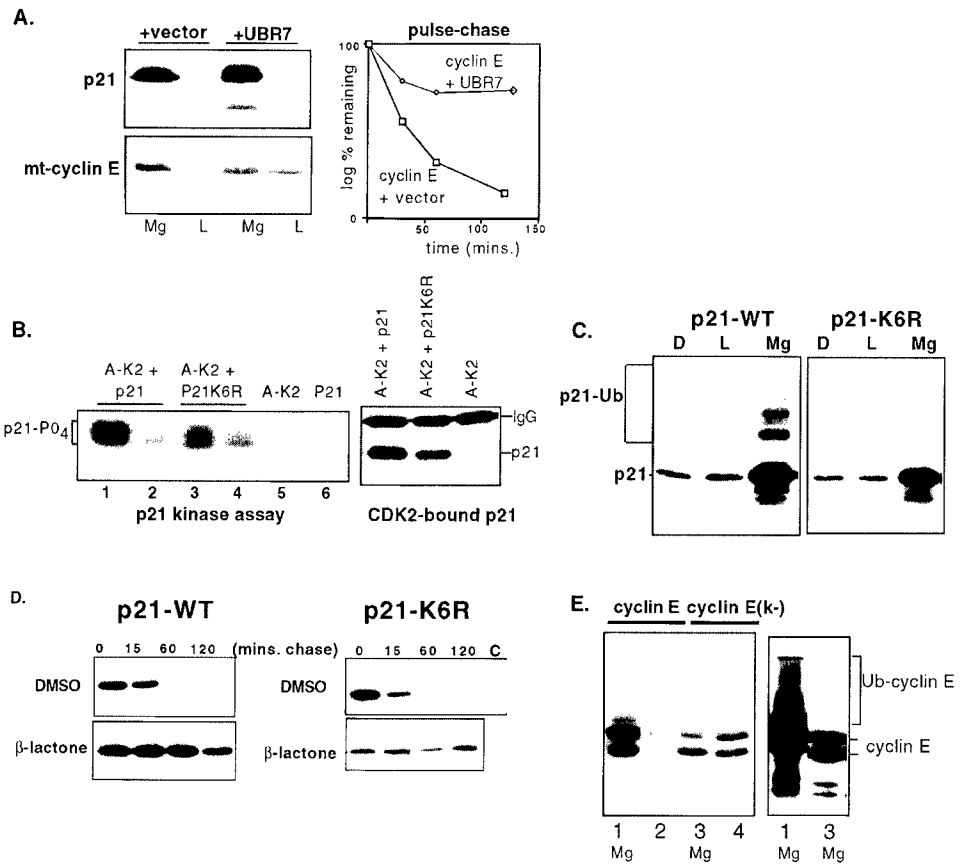

Figure 2. Inhibition of p21 Ubiquitination Does Not Affect Its Turnover

(A) 293 cells were cotransfected with either p21 or cyclin E and either empty vector or lysineless ubiquitin (UBR7). Cells were treated with either the control peptide aldehyde LLM (L) or MG-132 (Mg). Pulse–chase: half-life of transfected cyclin E ± UBR7 in 293 cells. To minimize possible effects of UBR7 on cyclin E turnover other than ubiquitination, cyclin E(R130A) was used because the turnover of this protein is not regulated by Cdk2 binding or phosphorylation (Clurman et al., 1996).

(B) p21K6R binds and inhibits cyclin A-Cdk2. 293 cells were transfected with 0.5 μg (lanes 1 and 3) or 2 μg (lanes 2 and 4) of p21 or p21K6R, 5 μg of CS2myc-cyclin A, and 3 μg of CMV-Cdk2 as indicated. Lysates were immunoprecipitated with anti-myc tag and than subjected to either a p21 kinase assay (left) or anti-p21 Western blot (right).

(C) Lysineless p21 is not ubiquitinated but remains proteasome dependent. NIH3T3 cells were transfected with either p21 or p21K6R and treated with DMSO (D), LLM (L), or Mg-132 (Mg).

(D) The half-lives of p21K6R and wt-p21 are similar. 293 cells were transfected with either CS2p21 or CS2p21K6R and treated with β-lactone as indicated.

(E) Lysineless cyclin E is stable and does not form ubiquitin conjugates. Left, 293 cells were transfected with vectors expressing either cyclin E or cyclin EΔK. Mg-132 treatment is indicated. Right, overexposure of lanes 1 and 3.

of human and mouse p21 (see Experimental Procedures). We directly compared the abundance of the exogenous wt-p21 to endogenous mouse p21 in the same cell population and found that the exogenous p21 was expressed at levels equal to or less than endogenous p21 (Figure 3A). We next observed that the exogenous wt-p21 and p21K6R were expressed at similar levels in the respective cell pools and that the infected cells proliferated normally, consistent with the observed levels of expression (Figures 3A–3C and data not shown). Furthermore, the retroviral wt-p21 and p21K6R bound to similar amounts of endogenous Cdk2, indicating that both proteins were incorporated into Cdk–complexes equivalently (Figure 3B).

We then measured turnover of the retrovirally expressed p21 and p21K6R in these stable cell lines. Treatment of cell pools expressing either wt-p21 or p21K6R with Mg-132 or β-lactone increased the steady-state accumulation of both proteins as assayed by Western blotting and by indirect immunofluoresence (Figures 3C and 3E and data not shown). Additionally, the half-lives of p21K6R and endogenous mouse p21 were almost indistinguishable within the same cells (Figure 3D). Thus, the turnover of p21K6R mirrored that of wild-type p21 under near-physiologic expression conditions.

Fourth, in rare cases, ubiquitin can be conjugated to proteins via the N-terminal α-NH2 group, although most mammalian proteins are N^{α} acetylated, and their N termini cannot be ubiquitinated (Brown, 1979; Hershko et al., 1984; Tsunasawa and Sakiyama, 1984). The N terminus was recently identified as the key ubiquitination site for the MyoD protein (Breitschopf et al., 1998). An important distinction between MyoD and p21 is that lysineless MyoD is ubiquitinated in vivo, whereas

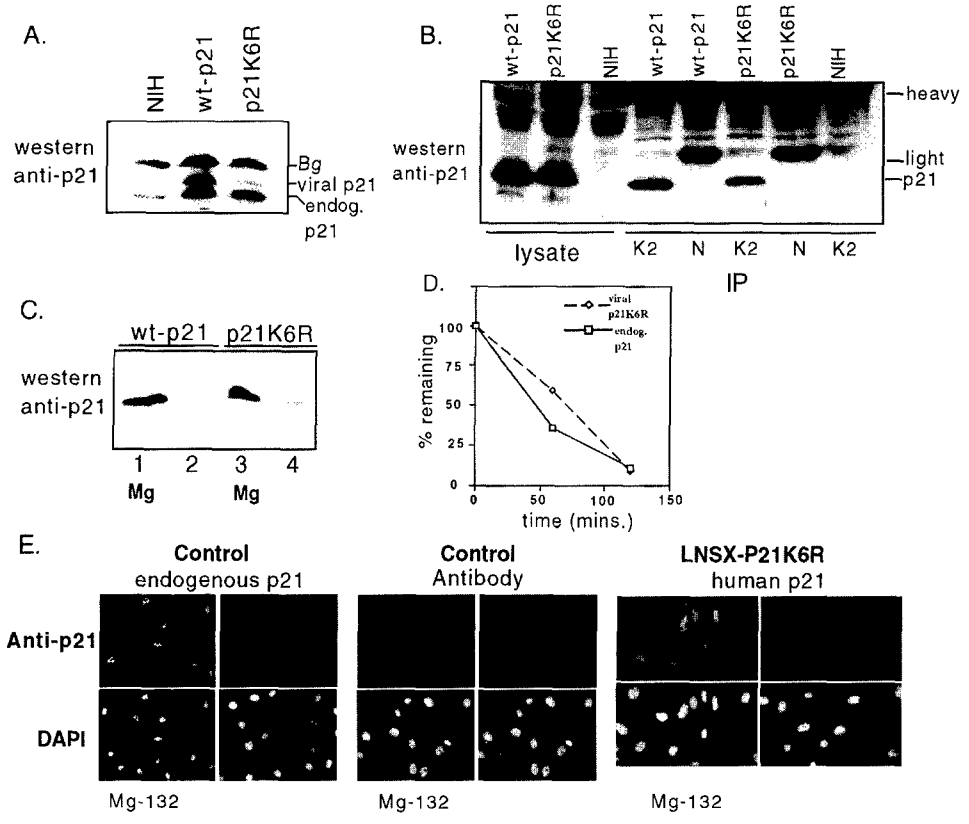

Figure 3. p21K6R Remains Proteasome Sensitive at Physiologic Levels of Expression

(A) NIH 3T3 cells transduced with LNSX-p21 and LNSX-p21K6R and control NIH3T3 cells were analyzed with polyclonal anti-p21(C-19), which recognizes exogenous p21 (human origin) and endogenous murine p21. Bg, background band. This antibody detects p21K6R poorly, but monoclonal anti-p21 (Transduction Labs) detects similar amounts of p21 and p21K6R in (B) and (C).

(B) Retrovirally expressed p21 and p21K6R are incorporated into Cdk-containing complexes. Cell lysates were immunoprecipitated with nonimmune serum (N) or anti-Cdk2 antibody (K2), followed by Western analysis with monoclonal anti-p21 (Transduction Labs).

(C) Retrovirally expressed wt-p21 and p21K6R are proteasome sensitive. The indicated cells were treated with Mg-132, followed by Western analysis with monoclonal anti-p21 (Transduction Labs).

(D) The half-lives of retroviral p21K6R and endogenous p21 are similar.

(E) Control NIH3T3 cells were stained with polyclonal anti-p21(C-19) (left panel), or monoclonal anti-p21 (Transduction Labs) (middle panel). LNSX-p21K6R cells were stained with monoclonal anti-21 (Transduction Labs) (right panel). Mg-132 treatment is indicated. The endogenous mouse p21 is detected by polyclonal but not monoclonal anti-p21 and is sensitive to proteasome inhibition. The exogenous human p21K6R is detected by monoclonal anti-p21 and is similarly responsive to proteasome inhibition.

p21K6R is not. Additional evidence that N-terminal ubiquitination is not important for p21 turnover was obtained by N-terminal epitope tagging p21 and p21K6R. This manipulation was previously shown to stabilize MyoD in vivo by preventing its amino-terminal ubiquitination (Breitschopf et al., 1998). However, the abundance and half-life of the N-terminal p21 fusion proteins remained sensitive to proteasome inhibition (Figures 4A and 4B). Furthermore, the N termini of human p21, murine p21, myc-p21 and HA-p21 (Met-Ser, Met-Ser, Met-Gly, and Met-Ala, respectively) all contain residues most frequently found in N-acetylated proteins (Tsunasawa and Sakiyama, 1984; Bradshaw et al., 1998). Therefore, N-terminal ubiquitin conjugation is unlikely to be the mechanism of p21 turnover since deletion of the p21 lysines eliminates p21–ubiquitin conjugate formation, indicating that ubiquitin is not attached to another site on the protein, and since the abundance and stability of N-terminal p21 fusion proteins remained sensitive to proteasome inhibition.

Regulation of p21 Turnover

We have begun to address the mechanisms that might direct p21 to the proteasome, if it is not via direct ubiquitination. These experiments have revealed that proteasomal turnover of p21 depends upon its localization to the nucleus. A p21 C-terminal truncation mutant lacking its nuclear localization signal, p21 (1–141), differed from wild-type p21 in two respects; it accumulated almost exclusively in the cytoplasm (Figure 4C), and its abundance was largely insensitive to proteasome inhibition (Figure 4D). To address the possibility that deletion of the C-terminal 25 amino acids removed an "instability" sequence, we added an SV40 NLS to p21(1–141) to redirect it to the nucleus. The readdition of an NLS effectively

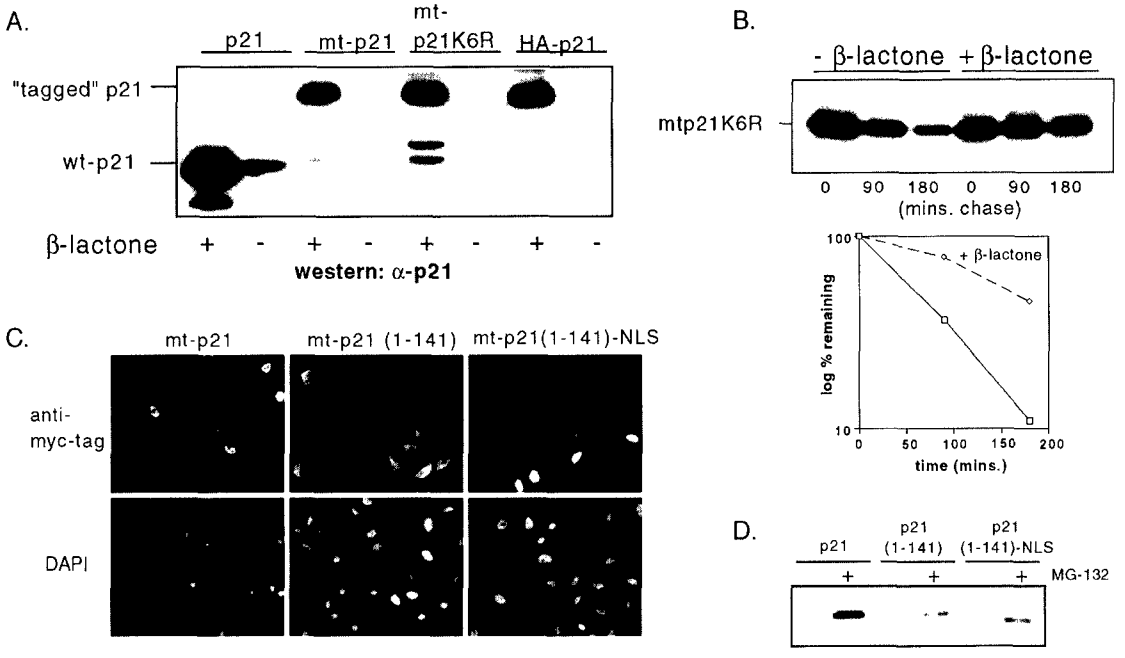

Figure 4. Proteasomal Regulation of N-Terminal p21 Epitope Tag Fusions

(A) 293 cells were transfected with expression vectors for the indicated epitope-tagged proteins and treated with β-lactone as shown.

(B) Pulse–chase analysis of mt-p21K6R ± β-lactone.

(C) A C-terminal deletion of p21 renders the protein cytoplasmic, and the addition of an SV40 NLS redirects the protein to the nucleus. HeLa cells were transfected with vectors expressing either mt-p21, mt-p21(1–141), or mt-p21(1–141)-NLS stained with anti-myc-tagged antibody.

(D) NIH3T3 cells were transfected with either CS2p21, CS2p21(1–141), or CS2p21(1–141)-NLS and treated with Mg-132 as indicated.

retargeted p21 (1–141) to the nucleus, and the protein regained its proteasome sensitivity.

In these respects, the turnover of p21 differs markedly from its close relative p27Kip1, which has been shown to be degraded in the cytoplasm. Furthermore, the nuclear export of transfected p27 is blocked by both proteasome inhibitors and the nuclear export inhibitor leptomycin B, thereby sequestering p27 in a cellular compartment where it is relatively stable (the nucleus) (Tomoda et al., 1999). In contrast, we find that p21 is degraded in the nucleus but is relatively stable in the cytoplasm. Furthermore, we have not observed any effect of leptomycin B on the abundance of transfected p21 (data not shown). Thus, unlike p27, proteasome inhibition does not indirectly stabilize p21 by restricting it to a cellular compartment where it is stable.

Discussion

p21 is an unstable protein exhibiting proteasome-sensitive turnover and ubiquitination in vivo. However, we have shown that p21 remains unstable and proteasome dependent even when it cannot be ubiquitinated. Therefore, direct p21–ubiquitin conjugates are not obligatory intermediates in proteasome-dependent p21 turnover. These data do not exclude the possibility that p21 turnover may be mediated by ubiquitination in some physiologic contexts. Alternatively, p21 ubiquitination may serve some function other than signaling proteolysis. Regardless, the example of p21 illustrates that observing multiubiquitination and proteasome sensitivity in vivo are insufficient to conclude that protein turnover must proceed through a ubiquitinated intermediate.

How might p21 be degraded by the proteasome independently of ubiquitin attachment? The clearest example of a protein whose turnover by the proteasome is ubiquitin independent is ornithine decarboxylase (ODC), and additional examples have been proposed (Murakami et al., 1992; Jariel-Encontre et al., 1995; Yu et al., 1997). ODC is directed to the proteasome by its specific binding partner, antizyme. Similarly, the interaction of p21 with its known binding partners affects its turnover, although these relationships are complex and poorly understood (Cayrol and Ducommun, 1998; Rousseau et al., 1999; unpublished observations). Perhaps the simplest explanation is that nonubiquitinated p21 is directly recognized by the proteasome. Unstructured proteins can be directly recognized and degraded by proteasomes in vitro and in vivo without ubiquitination (Katznelson and Kulka, 1985; Wenzel and Baumeister, 1993; Michalek et al., 1996). Moreover, free p21 does not have a well-defined tertiary structure (Kriwacki et al., 1996, 1997). Thus, p21 and perhaps other small Cdk inhibitors might be directly recognized by the proteasome when they are free of cyclin/Cdk complexes and adopt unstructured conformations.

The presence of ubiquitinated p21 suggests that p21 is associated with ubiquitinating enzymes. An interesting possibility is that p21 may be recruited to the proteasome by being bound to a protein that is itself ubiquitinated. Components of the SCF, an E3 ubiquitin ligase that regulates the abundance of G1 cyclins and Cdk

inhibitors in yeast, are themselves ubiquitinated (Zhou and Howley, 1998; Galan and Peter, 1999), and mammalian homologs of SCF proteins have been identified (Peters, 1998; Koepp et al., 1999). In fact, p21 has been reported to physically interact with complexes containing SCF proteins, and antisense inhibition of SCF proteins leads to p21 overaccumulation in vivo (Yu et al., 1998; Yam et al., 1999). Thus, perhaps p21 is recruited to the proteasome through its physical association with a ubiquitinated E3 complex.

Finally, we cannot exclude the possibility that proteasome inhibitors affect p21 turnover indirectly. For instance, proteasome inhibition may indirectly stabilize p21 by affecting protein(s) that regulate p21 abundance. Thus, p21 might be stable when bound to a protein that is itself proteasome dependent, or a p21 protease may be regulated by the proteasome.

Defining the role of the ubiquitin–proteasome system in protein turnover is complicated by its involvement in diverse biological processes. In the case of p21, our data support the notion that p21 turnover is regulated by the proteasome but indicate that this does not require direct p21 ubiquitination. The relative importance of direct ubiquitination in the degradation of other proteins, particularly mammalian proteins that have been analyzed only in vivo, remains a difficult issue. In some cases, the role of specific lysine residues has been directly demonstrated (examples include Scherer et al., 1995; Baldi et al., 1996; King et al., 1996b; Rodriguez et al., 1996; Yu et al., 1997). More commonly, the relative importance of direct ubiquitination in proteasomal protein degradation has not yet been determined.

Experimental Procedures

Cell Culture and Transfections

Human diploid fibroblasts were provided by C. Grandori (Seattle, WA); NIH3T3 and 293 cells were previously described (Sheaff et al., 1997). All cell lines were grown and transfected as described previously (Clurman et al., 1996). In most experiments, DNA concentration was 6 μg/60 mm dish. Different experimental conditions are noted. For wt-p21, p21-K6R, and p21 (1–141), NIH 3T3 cells and 293 cells were transfected with 2 μg and 1 μg of expression vector/60 mm dish, respectively, whereas 250 ng/60mm dish of mt-p21, mt-p21K6R, and HA-p21 vectors was used.

Plasmids, In Vitro Mutagenesis, and Retroviral Vectors

Plasmids were obtained from: pHA-ubiquitin (M. Treier, Heidelberg, Germany); p-CMV-CDK2 (E. Harlow, Charlestown, MA); pUBR7 (T. McGarry, R. King, and M. Kirschner, Boston, MA); pUNI15, pHM200-myc3, and pHM200-HA3 (S. Elledge, Houston, TX); and pLNSX (D. Miller, Seattle, WA). pCS2hp21, pCS2mt-cyclin E (M130A), pCS2mt-cyclin E, and pCS2mt-cyclinA were previously described (Clurman et al., 1996). CS2-p21K6R and CS2-MTEΔK were constructed from pCS2p21 and pCS2m-cyclin E by converting all of the lysine residues in each protein to arginine as described (Clurman et al., 1996). Primer sequences are available upon request.

For LNSX-p21 and LNSXp21K6R viral stocks, the CS2 p21 and p21K6R inserts were subcloned into the retroviral vector pLNSX (Miller and Rosman, 1989). Retroviral supernatants were prepared by transfecting the producer line Phoenix-Eco (obtained from G. Nolan, Palo Alto, CA.) with pLNSX-p21 and pLNSX-p21K6R and harvesting supernatants after 48 hr. 500,000 NIH3T3 cells were transduced with 3 ml of viral supernatant and selected with G418 (3 mg/ml).

The HA3-p21, HA3p21K6R, mt3p21, and mt3p21K6R constructs were made with the Univector system as described (Liu et al., 1998). The cDNAs of human p21 and p21K6R were subcloned into pUNI15 and recombined with either pHM200-myc3 or pHM200-HA3. p21(1–141) and p21(1–141NLS) were produced by generating PCR fragments beginning with the p21 ATG and ending at either residue 141, or adding KKKRKV (corresponding to the SV40 NLS) after residue 141, and subcloned into pCS2 or pCS2MT (myc-tagged) and sequenced.

Antibodies

The following antibodies were used: monoclonal anti-p21 (Transduction Labs); polyclonal anti-p21 antibody (C19), monoclonal anti-p21 antibody (F5), and anti-Cdk2(M2), (Santa Cruz Biotechnology); monoclonal anti-human cyclin E HE12 (Pharmingen), HRP-conjugated anti-mouse and anti-rabbit IgG (Amersham), FITC-anti mouse IgG, or anti-rabbit IgG (Jackson Labs). 9E10 (anti-myc tag) was prepared by an in-house facility.

Polyclonal anti-p21(C-19) detects both mouse and human p21 and was used to directly compare the expression of retrovirally expressed wild-type human p21 and endogenous mouse p21. Because the epitope recognized by this antibody contains lysines, it detects p21K6R poorly (see Figure 3A). Monoclonal p21 (Transduction Labs), which recognizes only human p21, was thus used to compare the level of wt-p21 to p21K6R expression and allows an indirect comparison of p21K6R and endogenous p21 expression.

Inhibitors

LLM (a control peptide aldehyde that does not inhibit the proteasome), Mg-132 (Calbiochem), and β-lactone (Boston Biochemical) were dissolved in DMSO. Approximately 24 hr after transfection, cells were treated with inhibitors overnight (LLM, 50 μM; MG-132, 2 μM; β-lactone, 10 μM). Leptomycin B was a gift of M. Yoshida (Tokyo) and used at 10 ng/ml.

Western Blotting, Immunoprecipitation, and Kinase Assays

Cells were lysed in RIPA buffer (10 mM tris [pH 7.4], 0.15 M NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 10 μg/ml each of aprotonin, leupeptin, and pepstatin, 50 mM NaF, 1 mM Na vanadate), followed by scraping and sonication. Lysates for coimmunoprecipitation were made in NP-40 buffer (0.5% NP-40, 20 mM Tris [pH 7.4], 150 mM NaCl). Cell extracts were electrophoresed on 12%–17% polyacrylamide gels and Western blotted was as described (Clurman et al., 1996). For immunoprecipitation, cell lysates normalized for protein concentration were processed as described (Clurman et al., 1996). For the p21 kinase assay, after the last wash, immunoprecipitates were resuspended in 20 μl kinase buffer containing 1 μm ATP + 0.5 μl [γ-^{32}P]ATP (6000 Ci/mM, NEN) and incubated at 37°C for 30 min.

Pulse–Chase

For endogenous p21, cells in 100 mm dishes were labeled with Tran^{35}label (ICN) at 500 μCi/ml in 2.2 ml DME without methionine/cysteine (ICN) for 15 min at 37°, then chased with DME/10% FBS with 400 mg/L methionine. Cells were lysed in RIPA and immunoprecipitated with polyclonal anti-p21 antibody (C-19) after preclearing. Modifications for transfected p21 were as follows: first, cells were transfected on 70%–80% confluent 100 mm dishes, and each dish was split into 6–8 60 mm dishes so that each time course was derived from the same transfection; second, 200 μCi/ml of label was used, and half of each 60 mm plate lysate immunoprecipitated with monoclonal anti-p21 (Transduction Labs). For pulse–chase analyses after proteasome inhibition, cells were treated with MG-132 or β-lactone for 2 hr prior to labeling, and inhibitors were present throughout all subsequent stages. Pulse–chase analyses of NIH-LNSXp21 and NIH-LNSXp21K6R were performed as described for endogenous p21, and lysates immunoprecipated with monoclonal anti-p21 (Transduction Labs) or monoclonal anti-p21 F5 (Santa Cruz).

Indirect Immunofluoresence

NIH3T3 or HeLa cells were transfected as above with glass coverslips in the dish. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS, then stained with primary antibody followed by FITC-anti-mouse IgG or FITC-anti-rabbit IgG (Jackson Labs). The final wash contained DAPI to visualize nuclei.

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